Jasmonate-dependent modifications of the pectin matrix during potato development function as a defense mechanism targeted by *Dickeya dadantii* virulence factors

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SUMMARY

The plant cell wall constitutes an essential protection barrier against pathogen attack. In addition, cell-wall disruption leads to accumulation of jasmonates (JAs), which are key signaling molecules for activation of plant inducible defense responses. However, whether JAs in return modulate the cell-wall composition to reinforce this defensive barrier remains unknown. The enzyme 13–allene oxide synthase (13–AOS) catalyzes the first committed step towards biosynthesis of JAs. In potato (*Solanum tuberosum*), there are two putative *St13–AOS* genes, which we show here to be differentially induced upon wounding. We also determine that both genes complement an Arabidopsis *aos* null mutant, indicating that they encode functional 13–AOS enzymes. Indeed, transgenic potato plants lacking both *St13–AOS* genes (*CoAOS1/2* lines) exhibited a significant reduction of JAs, a concomitant decrease in wound-responsive gene activation, and an increased severity of soft rot disease symptoms caused by *Dickeya dadantii*. Intriguingly, a hypovirulent *D. dadantii pel* strain lacking the five major pectate lyases, which causes limited tissue maceration on wild-type plants, regained infectivity in *CoAOS1/2* plants. In line with this, we found differences in pectin methyl esterase activity and cell-wall pectin composition between wild-type and *CoAOS1/2* plants. Importantly, wild-type plants had pectins with a lower degree of methyl esterification, which are the substrates of the pectate lyases mutated in the *pel* strain. These results suggest that, during development of potato plants, JAs mediate modification of the pectin matrix to form a defensive barrier that is counteracted by pectinolytic virulence factors from *D. dadantii*.

Keywords: jasmonic acid, OPDA, allene oxide synthase, cell wall, pectin methyl-esterification, *Dickeya*.

INTRODUCTION

The cell wall is a strong physical barrier that protects plant cells from pathogen infection and damage. This cellular structure was previously considered as an inert obstacle, but in the recent years has received attention as an integral part of the signaling pathways that are activated in a coordinated manner to trigger a successful plant defense response (Hematy *et al.*, 2009; Wolf *et al.*, 2012).

The primary cell wall of plants consists of a rigid scaffold of cellulose fibers embedded in a matrix of hemicellulose and pectins that glues the structure together and maintains cell-wall integrity and porosity. The pectin network serves as a site for recognition of molecules that warn of pathogen attack (Willats *et al.*, 2001; Peaucelle *et al.*, 2012), and also allows binding of specific cell wall-related enzymes, thus limiting their activities to defined regions of the wall. Disruption of cell-wall integrity by mechanical damage or pathogen attack not only triggers deposition of callose, a β-1,3-glucan that may act as an additional physical barrier

(Voigt and Somerville, 2009), but also releases oligogalacturonides from the pectin matrix. These oligogalacturonides participate as signaling molecules in the local defense (Doares *et al.*, 1995; Ridley *et al.*, 2001), activating jasmonic acid (JA)-dependent and -independent responses (Rojo *et al.*, 1999).

JA, its precursor 12–oxo-phytodienoic acid (OPDA), and the bioactive derivative jasmonate isoleucine (JA–Ile), collectively referred to as jasmonates (JAs), are key compounds in the regulation of plant responses to wounding and pathogen attack, not only in the damaged tissue but also systemically in distant, non-damaged tissues (Browse and Howe, 2008; Koo *et al.*, 2009). Mechanical damage induces rapid production of JAs, which activate their own biosynthetic genes via a positive feedback loop, producing high levels of JAs that trigger specific defense responses (Wasternack, 2007; Browse, 2009). Synthesis of JAs occurs through the 13–lipoxygenase (LOX) pathway, and requires oxygenation of linolenic acid by a 13–LOX to produce a 13–hydroperoxide that is converted to OPDA by simultaneous and sequential action of 13–allene oxide synthase (13–AOS) and allene oxide cyclase (AOC) (Schaller and Stintzi, 2009). Subsequent reduction of OPDA by a NADPH-dependent OPDA reductase (OPR3), CoA esterification of the carboxyl group (Koo *et al.*, 2006) and three cycles of β–oxidation yield JA (Schaller and Stintzi, 2009), which may be conjugated with amino acids, particularly isoleucine, by Jasmonic Acid Amido Synthetase (JAR1) (Staswick and Tiryaki, 2004), giving rise to the biologically active compound. Formation of the unstable allene oxide intermediate 12,13–(*S*)-epoxy-octadecatrienoic acid by 13–AOS constitutes the first committed step in the JA biosynthetic pathway. In potato (*Solanum tuberosum* L.), two genes, *StAOS1* (Sotub04g032980) and *StAOS2* (Sotub11g026710), encode putative 13–AOS enzymes, and a third, *StAOS3* (Sotub10g008380), encodes a potential 9/13–AOS. Potato AOS3 preferentially acts on 9–hydroperoxides of linoleic and linolenic acids in below-ground organs, and is not involved in JA synthesis (Stumpe *et al.*, 2006). In contrast, AOS2 was shown to restore JA synthesis upon transformation into the *Arabidopsis thaliana aos* mutant (Pajerowska-Mukhtar *et al.*, 2008). Moreover, *StAOS2* associates with potato QTL markers for resistance to late blight disease caused by *Phytophthora infestans* (Pajerowska *et al.*, 2005), and specific *StAOS2* alleles associated with resistance to this pathogen have been identified (Pajerowska-Mukhtar *et al.*, 2008).

The observation that plants with mutations in genes involved in cell-wall biosynthesis have altered JA responses revealed a close and largely unexpected relationship between cell-wall structure and JA signaling. Indeed, the *cev1* mutant, in which the cellulose synthase CeSA3 is affected (Ellis *et al.*, 2002), and the *cob* mutant, in which cellulose deposition is affected (Ko *et al.*, 2006), show constitutive expression of stress response genes and increased production of JA. However, the implications of the cell wall-JAs relationship in plant development and defense responses have not been studied in detail. For instance, it is not known whether cell-wall synthesis is influenced by JAs and whether changes in the content of JAs provoke modifications of the cell wall that may influence defense responses.

A combined understanding at the molecular and metabolic levels of the mechanisms that regulate the content of JAs is essential for improving plant tolerance to biotic stress. Here, we report that depletion of 13–AOS in potato reduces OPDA, JA and JA–Ile content upon wounding, modifies cell-wall composition, and increases susceptibility to the bacterial pathogen *Dickeya dadantii*, the causal agent of soft rot. Our results suggest that JAs regulate modification of cell-wall pectins under non-challenged conditions, leading to a cell-wall structure that is less susceptible to action of the macerative capacity deployed by soft rot pathogens.

RESULTS

The rapid accumulation of JA–Ile does not rely on wound-induced expression of JA biosynthetic genes

Although it is well-documented that leaf wounding rapidly activates synthesis of JAs (Glauser *et al.*, 2008; Koo *et al.*, 2009), the temporary sequence and causal correlation between wound-induced expression of the biosynthetic genes and accumulation of the corresponding JAs has received less attention. To analyze the time course of this series of events in detail, we monitored the response of the JA biosynthetic pathway to wounding in potato plants. Expression of the 13–LOX pathway is significantly induced by wounding (Figure 1a and Figure S1a). However, a differential pattern was observed for *StAOS1* and *StAOS2* expression. Whereas *StAOS1* was specifically and rapidly expressed in damaged leaves, *StAOS2* showed a delayed induction that was more intense in the distal leaves (Figure 1a). mRNA accumulation for *StLOXH3*, which encodes the 13–LOX isoform that acts early in the JA biosynthetic pathway, was rapid and transient (Royo *et al.*, 1999), following a time course similar to *StAOS1* (Figure S1a). Induction of the downstream biosynthetic genes *StAOC*, *StOPR3* and *StJAR1* was also stronger in damaged leaves, predominantly 2 h after wounding, and was essentially absent in the distal tissues. These results indicate that there is a coordinated, but kinetically different, activation of the genes involved in production of JAs a short time after wounding. Moreover, the distinct time courses of induction suggest a possible differential role for the two 13–AOS present in potato.

To obtain further insight into the possible roles of the two *St13–AOS* genes in JA synthesis, the cell-specific

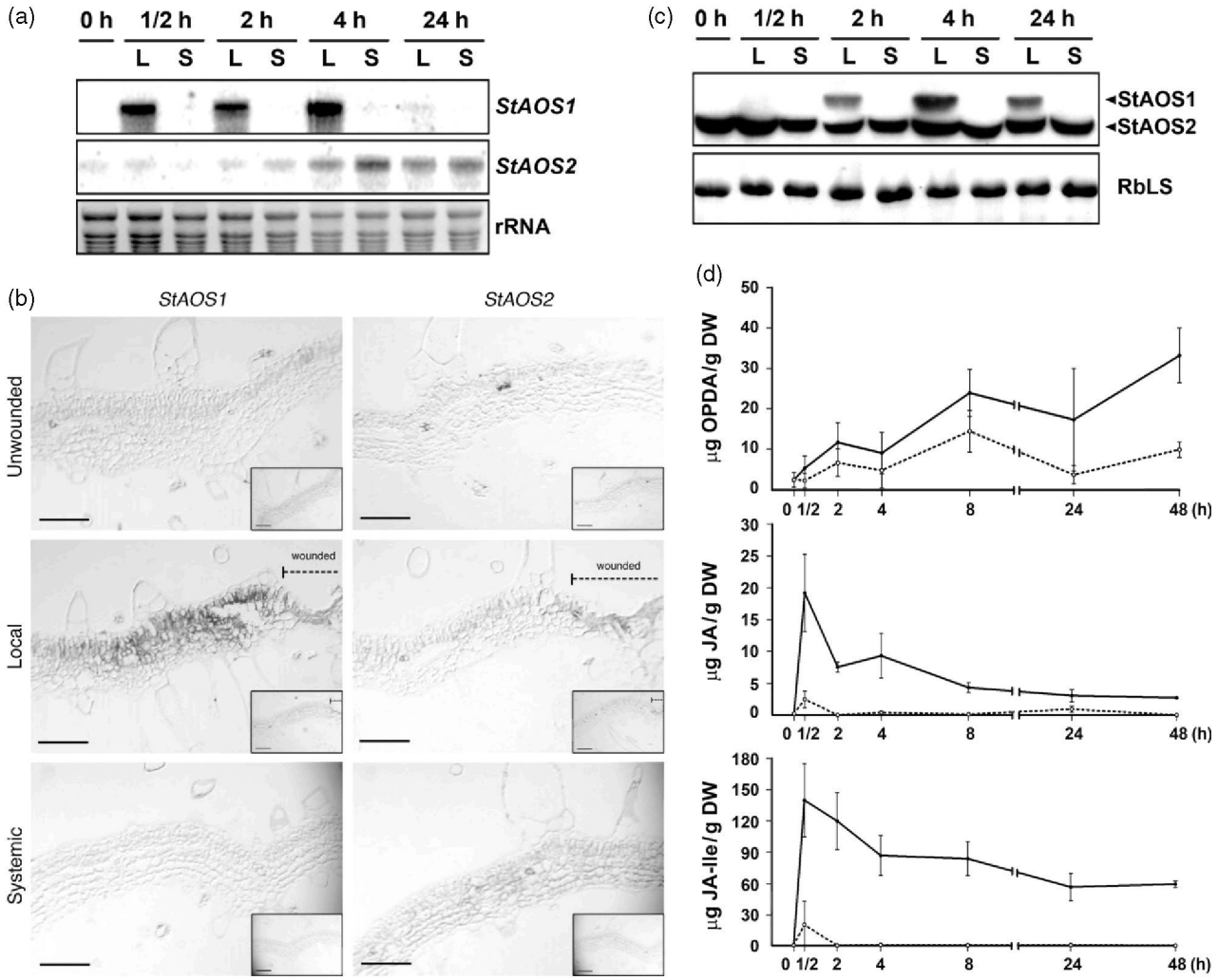

Figure 1. Wound response in potato leaves.

(a) Total RNA extracted from wounded (L) and systemically induced (S) leaves collected at the times after wounding indicated above the lanes was analyzed by Northern blot with *StAOS1*- and *StAOS2*-specific probes. Ethidium bromide staining of rRNA is shown as a loading control.

(b) *In situ* hybridization of non-wounded (top panel), 4 h damaged (middle panel) and systemically induced leaves (bottom panel) with either an *StAOS1* (left) or an *StAOS2* (right) antisense probe. Inset: sense probe. Scale bars = 100 µm.

(c) Levels of AOS1 and AOS2 in leaves as described in (a) were assayed by Western blotting with a specific antibody. Ponceau staining of the large subunit of Rubisco (RbLS) is shown as a loading control. The RNA and protein blots presented here are representative of four biologically independent experiments.

(d) OPDA, JA and JA–Ile content (µg per gram of dry weight) in wounded leaves (solid line) and systemically induced leaves (dashed line) collected at the indicated times after wounding. Values are means ± standard deviation of data from three biologically independent experiments.

distribution of *StAOS1* and *StAOS2* mRNAs was analyzed by *in situ* hybridization. Consistent with the results obtained from Northern blot analyses, *StAOS1* mRNA was detected in the damaged leaves, preferentially accumulating in the parenchyma close to the wound site. No *StAOS1* mRNA was detected in systemically induced, non-damaged leaves. Conversely, *StAOS2* mRNA was barely detected in the damaged leaves, whereas *StAOS2* mRNA accumulation upon wounding was observed in distal leaves (Figure 1b).

Variations in gene expression do not always result in equivalent changes in protein levels. We therefore determined the levels of the two AOS isoforms in leaves as a result of wound induction of their respective genes (Figure 1c). Confirming the gene expression data, AOS1 protein was only detected in damaged leaves, with a time course of accumulation that showed some delay compared with that of the corresponding mRNA. In contrast, AOS2 was easily detected in non-wounded leaves even though its mRNA was barely detectable. However, in spite of its transcriptional activation, AOS2 did not accumulate at much higher levels upon wounding, suggesting that its steady-state content is subject to additional post-transcriptional regulation. Thus, damaged leaves accumulate both AOS1 and AOS2, whereas AOS2 is the only isoform

present in either systemically induced non-damaged leaves or those from non-wounded plants.

We next determined the effect of wounding on the plant OPDA, JA and JA–Ile content. Wounding of potato plants elicited a progressive and continuous increase in OPDA, reaching a concentration in the damaged leaves at 48 h after wounding that was 13 times the basal level (Figure 1d). In non-damaged, systemically induced leaves, the OPDA level showed a steady increase to five times the basal content, attaining the highest concentration by 8 h after wounding. In contrast, the JA and JA–Ile content rapidly and sharply increased in damaged leaves, with a peak at 30 min after wounding of approximately 200 times the basal levels, as previously reported for other species (Glauser *et al.*, 2008; Koo *et al.*, 2009; Suza *et al.*, 2010). Moreover, the content of both compounds, JA–Ile in particular, remained substantially above the basal level for the period studied (Figure 1d). In the systemically induced leaves, a transient peak in the JA and JA–Ile content, much lower than that in damaged leaves, was consistently observed 30 min after wounding, later decreasing to basal levels (Figure 1d). These early peaks of JA and JA–Ile accumulation occur before induction of *StOPR3* and *StJAR1* expression (compare Figure 1d and Figure S1a), suggesting that the basal expression of these genes, albeit low, is sufficient to provide enough OPR3 and JAR1 activity for JA and JA–Ile synthesis when sufficient substrate is available. Substrate limitation may therefore act as a major check-point for initiating wound-induced synthesis of JA and JA–Ile, as previously suggested (Laudert *et al.*, 2000; Kubigsteltig and Weiler, 2003). After this rapid increase, feedback activation of the biosynthetic enzymes may also be a major factor in maintaining the JA and JA–Ile content above the basal level for an extended period. Indeed, although the levels of the precursor OPDA remain high in both wounded and distal tissues, continued accumulation of JA and JA–Ile occurs only in wounded tissues, coinciding with the specific activation of *StOPR3* and *StJAR1*.

To explore how these changes in the levels of signaling compounds correlate in time with the expression of defense response genes, we monitored the mRNA accumulation of early and late wound-responsive genes. *StMYC2*, a gene involved in the JA signaling pathway (Boter *et al.*, 2004; Lorenzo *et al.*, 2004), showed rapid induction upon wounding in both locally damaged and systemically induced leaves, but was more transient in the latter (Figure S1b). This expression pattern therefore mirrored the accumulation profile of JA and JA–Ile. In the case of proteinase inhibitor 2 (*PIN2*), leucine aminopeptidase (*LAP*) and threonine deaminase (*TD*), a slower increase in mRNA accumulation was detected in both damaged and distal leaves (Figure S1b), which may mirror the accumulation of OPDA in damaged and systemically induced tissues.

Simultaneous depletion of *StAOS1* and *StAOS2* expression reduces OPDA, JA and JA–Ile content

To specifically interfere with the synthesis of JAs, we targeted the 13–AOS enzymes that catalyze the first committed step in this pathway. To first ensure that both *StAOS1* and *StAOS2* encode active 13–AOS enzymes, we introduced *35S:StAOS1* or *35S:StAOS2* into the *Arabidopsis thaliana aos* mutant (Park *et al.*, 2002) (Figure 2a), and observed that the male-sterile phenotype was complemented (Figure 2b–e). Moreover, expression of either *35S:StAOS1* or *35S:StAOS2* in the *aos* background restores wound-induced expression of *VSP* (Figure 2f), a well-described marker for JA-dependent wound responses in Arabidopsis (Rojo *et al.*, 1999). Therefore, both potato AOS isoforms perform the same enzymatic function in the JA biosynthetic pathway during development and in response to wounding. Using the same strategy, we next generated transgenic potato plants with altered AOS1 and AOS2 levels. Over-expression of either *StAOS1* or *StAOS2* led to ectopic accumulation of high levels of either AOS1 or AOS2 but did not result in any significant change in wound-inducible gene expression (data not shown). In addition to these over-expressing plants, we also identified transgenic lines that were specifically depleted of each 13–AOS by co-suppression of the corresponding genes (*CoAOS1* and *CoAOS2*), and characterized their oxylipin profiles and wound-induced gene expression. Transgenic *CoAOS1* lines showed no accumulation of *StAOS1* mRNA in any of the tissues or under any conditions studied, including damaged leaves, while *StAOS2* expression was somewhat reduced. Conversely, *CoAOS2* lines showed a complete absence of *StAOS2* mRNA and slightly lower *StAOS1* expression upon wounding (Figure S2a). In both cases, co-suppression provoked a decrease in the basal OPDA level. Moreover, although OPDA content in the co-suppressed plants increased upon wounding, both types of transgenic lines presented significantly lower OPDA levels than the wild-type (WT). In contrast, both transgenic lines differed in the pattern of JA and JA–Ile accumulation. Despite the wound-induced expression of *StAOS1*, *CoAOS1* lines showed little variation compared to WT plants in JA and JA–Ile content in damaged leaves, whereas *CoAOS2* lines showed a significantly lower JA increase upon wounding (Table S1). To establish whether the wound response was affected in the co-suppressed lines, we analyzed *StPIN2* and *StTD* expression after wounding. *CoAOS1* and *CoAOS2* plants showed a slight reduction in expression of both genes, which was more evident in the distal leaves (Figure S2b). These results suggest that both AOS1 and AOS2 are required to attain the wild-type level of OPDA synthesis. However, although reduced, the OPDA content of the co-suppressed lines is enough to maintain JA and JA–Ile concentrations that are sufficient to drive a proper wound response.

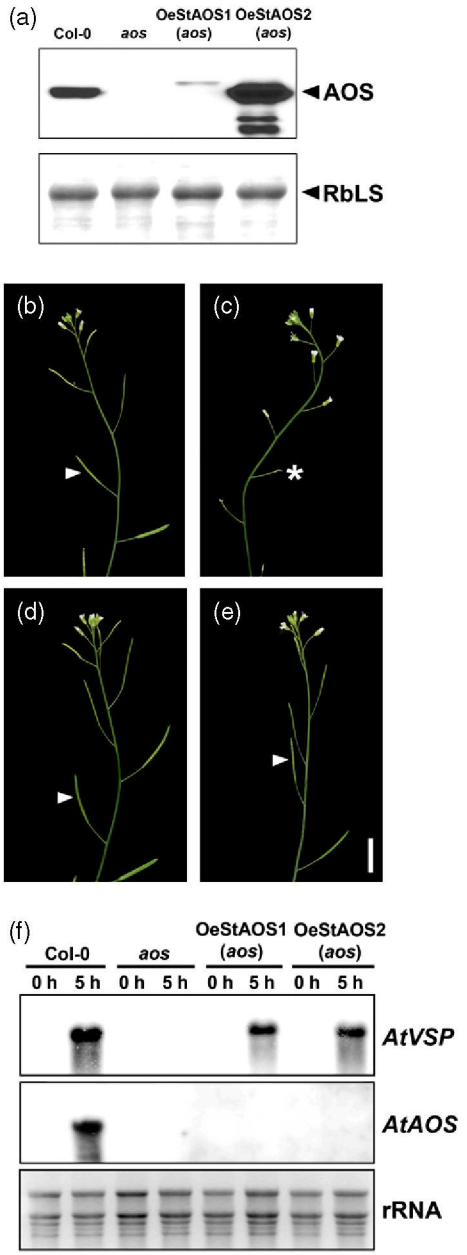

Figure 2. Potato AOS1 and AOS2 complement the Arabidopsis *aos* mutant.
(a) AOS levels were determined by Western blotting in 10-day-old Arabidopsis WT seedlings (Col–0), seedlings of the *aos* mutant, and seedlings over-expressing *StAOS1* [OeStAOS1 (*aos*)] or *StAOS2* [OeStAOS2 (*aos*)] in the *aos* background. Ponceau staining of the large subunit of Rubisco (RbLS) is shown as a loading control.
(b–e) Complementation of the *aos* male-sterile phenotype. (b) Normal siliques developed in Arabidopsis WT floral shoots (arrowhead). (c) Sterile *aos* floral shoot with aborted siliques (asterisks). (d,e) Fertile floral shoots of Arabidopsis plants over-expressing *StAOS1* (d) or *StAOS2* (e) in the *aos* mutant background. Arrowheads show normal siliques. Scale bar = 1 cm.
(f) *In vitro*-grown 10-day-old Arabidopsis WT (Col–0), *aos*, OeStAOS1 (*aos*) and OeStAOS2 (*aos*) seedlings were wounded, and accumulation of *AtVSP* and *AtAOS* transcripts was monitored by Northern blot analysis in non-wounded seedlings (0 h) and in seedlings 5 h after wounding. Ethidium bromide staining of rRNA is shown as a loading control.
The RNA and protein blots are representative of three biologically independent experiments.

We therefore reasoned that substantial modification of the JA and JA–Ile content may require simultaneous co-suppression of *StAOS1* and *StAOS2*, thus altering the wound response of the plant. To test this hypothesis, we generated potato plants in which both *StAOS1* and *StAOS2* (*CoAOS1/2*) were co-suppressed. Two independent lines showing a significant reduction in expression of both *StAOS1* and *StAOS2* genes (Figure 3a) were chosen for further characterization. OPDA content in the non-damaged leaves of one of these lines (*CoAOS1/2–A*) was 12 times lower than WT, and it was almost undetectable (90 times lower) in the other (*CoAOS1/2–B*) (Figure 3b). Upon wounding, there was essentially no increase in the OPDA content in either of the *CoAOS1/2* lines, except for a transient increase in the damaged leaves of *CoAOS1/2–A* 30 min after wounding that is nevertheless three times lower than the increase observed in WT leaves (Figure 3b). In distal leaves, there was also a small increase in OPDA that was seven times lower than in WT leaves (Figure 3c). The changes in OPDA level led to significant reductions in the content of bioactive JA–Ile, with a two- to three-fold decrease in damaged leaves at any time analyzed (Figure 3b). Interestingly, the variation in JA–Ile content was even more pronounced in the distal leaves, being 15 times lower than WT (Figure 3c). This reduction of bioactive JA–Ile was accompanied by a decrease in expression of the late wound-responsive genes *StPIN2* and *StTD*, which was more drastic in systemically induced leaves (Figure 3d).

Suppression of 13–AOS activity in potato provokes high susceptibility to soft rot disease caused by a hypovirulent *D. dadantii* strain

To test whether the modifications in the levels of JAs and the expression of wound-response genes were associated with altered resistance to pathogens, we analyzed the interaction with the natural potato pathogen *Dickeya dadantii*, the causal agent of soft rot disease. In Arabidopsis, JAs have been shown to play a positive role in defense against this pathogen (Fagard *et al.*, 2007; Antunez-Lamas *et al.*, 2009). This function appears to be conserved in potato, as we observed increased susceptibility to the pathogen in *CoAOS1/2* plants (Figure 4a–e). This suggests that AOS1 and AOS2, through production of JAs, activate physiologically relevant defense responses against *D. dadantii* in potato. We also included in these pathogen assays a hypovirulent *D. dadantii pel* strain lacking the five major pectate lyases (PelA, PelB, PelC, PelD and PelE), which play a central role in tissue maceration and pathogenicity (Beaulieu *et al.*, 1993). We assumed that, due to the impairment in cell-wall degradation, this strain would not infect potato plants, regardless of whether downstream JAs defenses were activated or not. As expected, disease progression was almost undetectable in WT plants inoculated with the

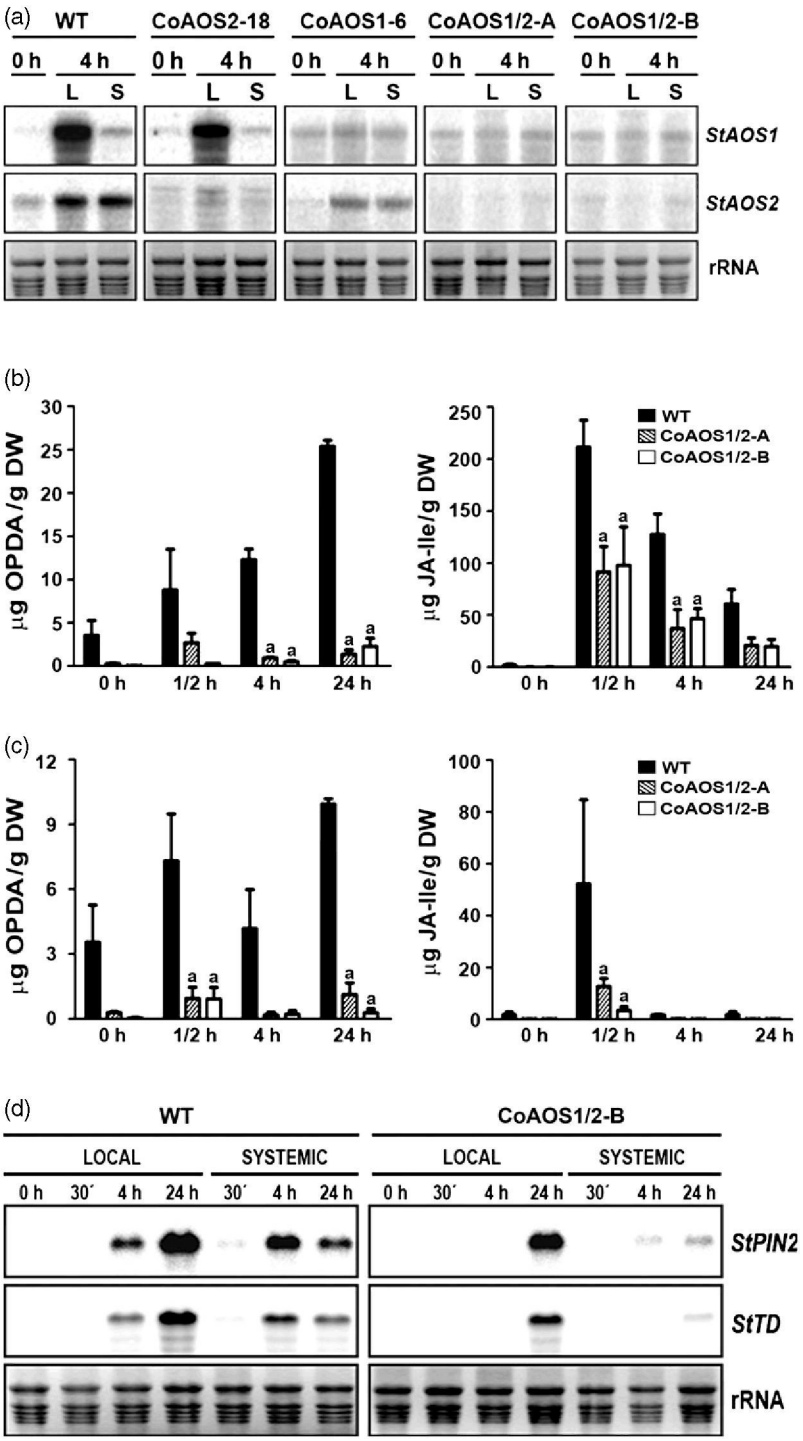

Figure 3. Characterization of wound responses in *CoAOS1/2* plants.

(a) Total RNA extracted from control (0 h), 4 h wounded (4 h, L) and systemically induced (4 h, S) leaves from wild-type (WT), single co-suppression (*CoAOS1–6* and *CoAOS2–18*), and double co-suppression (*CoAOS1/2–A* and *CoAOS1/2–B*) plants was analyzed by Northern blot using *StAOS1*- and *StAOS2*-specific probes. Ribosomal RNA staining (rRNA) was used as a loading control.

(b,c) OPDA and JA–Ile content of wounded (b) and systemically induced leaves (c) collected at the indicated times after wounding from WT (black), *CoAOS1/2-A* (hatched) and *CoAOS1/2-B* (white) plants. Values are means ± standard error of data from three biologically independent experiments. The letter 'a' above the bars indicates a significant difference (two-way ANOVA; $P < 0.05$) in *CoAOS1/2* samples compared with WT potato plants.

(d) Total RNA extracted from wounded (local) and systemically induced (systemic) leaves collected at the times after wounding indicated above the lanes was analyzed by Northern blot with *StPIN2*- and *StTD*-specific probes. Ethidium bromide staining of rRNA is shown as a loading control.

The blots presented are representative of four independent experiments.

D. dadantii pel mutant (Figure 4e,f), underscoring the importance of Pel-mediated hydrolysis for infection. However, infectivity of the *D. dadantii pel* strain was restored in *CoAOS1/2* plants, with levels of tissue maceration similar to those provoked by the *D. dadantii* 3937 strain in WT plants (Figure 4e). The extended stem maceration in *CoAOS1/2* plants caused bowing of the shoot and chlorosis of adjacent leaves, some of which became necrotic (Figure 4g). These results suggest that the remaining cell wall-degrading enzymes present in this mutant strain are sufficient to macerate *CoAOS1/2* but not WT tissues.

Suppression of 13–AOS activity in potato plants provokes large modifications in cell-wall composition

The unexpected high levels of tissue maceration caused by the *D. dadantii pel* strain on *CoAOS1/2* plants but not on WT suggest that suppressing 13–AOS activity leads to constitutive alterations in cell-wall composition. To validate this hypothesis, we studied cell-wall composition by FTIR spectrometry. Comparison of averaged spectra showed marked differences between WT and *CoAOS1/2* cell walls in fingerprint regions corresponding to carbohydrate absorbances between 900 and 1200 cm^{-1} (Figure 5a; arrowheads). Principal component analysis (PCA) of the FTIR spectra clearly separated WT and *CoAOS1/2* cell-wall populations (Figure S3a). We observed that much of the variation (90%) between WT and *CoAOS1/2* corresponded to changes at IR band intensities 1155, 1084, 1024 and 1004 cm^{-1} (Figure S3b), previously described as characteristic for pectin (Kacurakova *et al.*, 2000). To determine whether these changes were accompanied by alterations of the cell-wall sugar composition, we quantified the neutral monosaccharides and the galacturonic acid content in WT and *CoAOS1/2* plants. No significant differences were observed between these genotypes (Table S2). We subsequently used oligosaccharide mass profiling (Obel *et al.*, 2009) to perform a deeper characterization of WT and *CoAOS1/2* cell-wall pectins. WT potato leaf cell walls showed predominant ions at 804, 842, 852 and 868 *m/z* (Figure 5b). However, oligosaccharide mass profiling of *CoAOS1/2–A* and *CoAOS1/2–B* revealed a clear decrease in peak intensity at 852, with the peak at 868 *m/z* being barely detectable. Both peaks at *m/z* 852 and 868 presented the same MS/MS fragmentation, corresponding to sodium and potassium adducts of a rhamnogalacturonan oligosaccharide with a degree of polymerization of 5 (Figure 5b and Figure S4).

Interestingly, we also observed strong peak divergences in the FTIR regions from 1602 to 1614 cm^{-1} and at 1740 cm^{-1} (Figure 5a; arrows), which have been assigned to methyl esterification of pectin carbonyl groups (Kacurakova *et al.*, 2000, 2002; Mouille *et al.*, 2003), suggesting a higher degree of methyl esterification of the pectins of *CoAOS1/2* plants that may be due to lower pectin methyl esterase (PME) activity in these plants. To test this hypothesis, we determined the total PME activity in non-challenged leaves of WT and *CoAOS1/2* potato plants. Consistent with the composition of their respective cell walls, WT leaves have significantly higher PME activity than either *CoAOS1/2* transgenic line, which exhibited almost identical values (Figure 5c). Moreover, oligosaccharide mass profiling analysis of cell walls treated with polygalacturonase alone, thus cleaving de-methyl-esterifed homogalacturonan only, showed a higher amount of oligosaccharides extracted from WT cell walls (Figure S5). These results indicate that suppression of 13–AOS leads to reduced PME activity, in turn increasing the methyl esterification level of pectins in the cell wall. Hence, PMEs are likely targets for JA to mediate modifications in cell-wall composition. Importantly, the differences in pectin composition between WT and *CoAOS* plants explain their differential sensitivity to soft rot disease, particularly the weak symptoms caused by the *pel* strain in WT plants and the regained infectivity of this strain in *CoAOS* plants. These results provide strong evidence that JAs regulate cell-wall

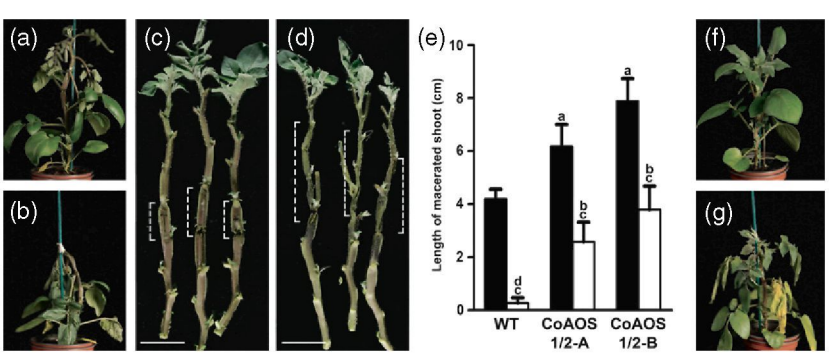

Figure 4. Infectivity of the *D. dadantii pel* mutant in *CoAOS1/2* plants.

(a,b) Symptoms on WT (a) and *CoAOS1/2* (b) plants 2 days after inoculation with *D. dadantii* 3937.

(c,d) Close-up views showing the maceration of stems of WT (c) and *CoAOS1/2* (d) plants 2 days after inoculation with *D. dadantii* 3937. WT stems showed a dark-brown color but remained largely turgid, whereas *CoAOS1/2* stems were almost completely macerated. Brackets indicate the macerated region. Scale bars = 5 cm.

(e) Mean macerated stem length 2 days after infection with *D. dadantii* 3937 (black) or *D. dadantii pel* mutant (white) of the indicated plant genotypes. Values are means from three independent experiments. The letter 'a' indicates significant differences (Student's *t* test; $P < 0.05$) in susceptibility to *D. dadantii* 3937 compared to WT potato plants. The letter 'b' indicates significant differences (Student's *t* test; $P < 0.05$) in susceptibility to *D. dadantii pel* compared with WT potato plants. The letter 'c' indicates significant differences (Student's *t* test; $P < 0.05$) in susceptibility to *D. dadantii pel* compared with the susceptibility to *D. dadantii* 3937 for each potato genotype. The letter 'd' indicates significant differences (Student's *t* test; $P < 0.05$) in susceptibility to *D. dadantii pel* compared with the susceptibility of WT plants infected with *D. dadantii* 3937.

(f,g) Symptoms on whole WT (f) and *CoAOS1/2* (g) plants 2 days after inoculation with the *D. dadantii pel* mutant.

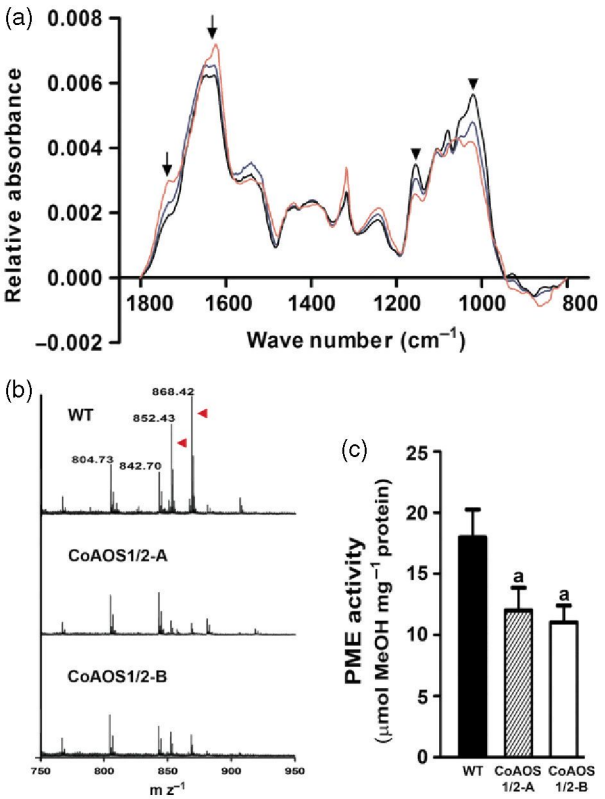

Figure 5. Cell-wall analysis of *CoAOS1/2* plants.

(a) Absorbance spectra from FTIR analysis of WT (black), *CoAOS1/2-A* (blue) and *CoAOS1/2-B* (red) cell walls. Arrowheads indicate regions characteristic of pectin (Kacurakova *et al.*, 2000). Arrows indicate regions associated with methyl esterification of pectin carbonyl groups.

(b) MALDI-TOF/MS analysis of pectic oligosaccharides after digestion of cell walls of WT (top), *CoAOS1/2-A* (middle) and *CoAOS1/2-B* (bottom) leaves. Arrowheads indicate significant peaks at 868.42 and 852.43 *m/z* that are not present in co-suppressed lines.

(c) PME activity was determined in leaves from WT (black), *CoAOS1/2-A* (hatched) and *CoAOS1/2-B* (white) plants. Values are means ± standard deviation from three biologically independent experiments. Statistically significant differences compared with WT as determined by one-way ANOVA ($P < 0.05$) are indicated by the letter 'a' above the bars.

composition, and suggest that this is a relevant component of the JA-dependent defense measures taken to avoid pathogen infection.

DISCUSSION

Upon attack by pests and/or pathogens, plants turn on inducible defense responses controlled by complex and finely tuned signaling pathways that enable effective transduction of these stress cues. A major wound-signaling pathway involves JAs, whose biosynthetic pathway is rapidly activated upon mechanical damage (this study; Farmer and Ryan, 1992; Strassner *et al.*, 2002; Wasternack, 2007). Perception of large and transient increases in the endogenous levels of JAs leads to transcriptional activation of an array of defense genes. Therefore, understanding how JA synthesis is controlled in response to wounding is crucial if the aim is to improve plant defense responses. Our results indicate that, in wounded leaves, there is a rapid induction of *StLOXH3* and *StAOS1*, which encode enzymes involved in the early steps of the 13–LOX pathway leading to synthesis of JAs. This enhanced transcription is mirrored by a progressive increase in the OPDA content, the first specific product in the JA biosynthetic route. However, the increase in its derivatives JA and JA–Ile that also ensues upon wounding precedes the induction of *StOPR3* and *StJAR1* expression (this study; Koo *et al.*, 2009; Suza *et al.*, 2010; Suza and Staswick, 2008), suggesting that the basal levels of these enzymes are sufficient to produce enough JA and JA–Ile when OPDA is available. Thus, OPDA availability is probably one of the check-points in wound-induced production of bioactive JA–Ile. The rapid and specific accumulation of AOS1 in wounded leaves reinforces this idea, and suggests that its activity may be required to maintain proper OPDA levels to allow a correct local response to mechanical damage, whilst AOS2 activity may largely account for OPDA production in non-challenged plants. In addition, activation of specific lipases that release OPDA from membrane galactolipids (Kourtchenko *et al.*, 2007) and/or of transport of this compound from the chloroplast to the peroxisomes, where it is further processed by OPR3, may contribute to provision of a sufficient supply of OPDA for JA synthesis.

To further analyze the regulatory points in the biosynthesis of JAs, we have genetically modified the content of OPDA, JA and bioactive JA–Ile in potato leaves by co-suppressing the two *St13–AOS* genes, encoding the first enzyme specifically committed to synthesis of JAs. Individual co-suppression suggested that AOS2 was the main 13–AOS responsible for the basal level of OPDA (Pajerowska-Mukhtar *et al.*, 2008), and may act as a reservoir to facilitate the response to unanticipated damage and/or stress conditions, a hypothesis consistent with the abundance of AOS2 observed in non-damaged plants. Although it has been suggested that accumulation of JAs in potato depends exclusively on AOS2 (Pajerowska-Mukhtar *et al.*, 2008), involvement of AOS1 in the JA biosynthetic pathway had not yet been experimentally determined. Our results indicate that, in order to significantly reduce the JA–Ile content, it is necessary to reduce the level of OPDA by simultaneously depleting both 13–AOS enzymes. This depletion leads to an alteration in wound-induced gene expression, with these changes being particularly obvious in the systemically induced leaves. Moreover, both AOS1 and AOS2 are able to complement the male sterility caused by the *aos* mutation in Arabidopsis, and to simultaneously restore wound-induced gene expression, indicating that both isoforms are active in OPDA synthesis. In Arabidopsis, OPDA content also plays an important role in control of systemic wound signaling, and it was suggested that an enzyme preceding JAR1 may regulate systemic JA–Ile

production (Koo *et al.*, 2009). Therefore, our findings suggest that JA–Ile production may largely be dependent on AOS activity, and that both AOS1 and AOS2 isoforms are required for expression of wound response genes in systemically induced tissues.

JAs are essential to trigger the plant defense response against necrotrophic pathogens, such as *D. dadantii*. These pathogens secrete large amounts of enzymes to degrade the plant cell wall and obtain nutrients, resulting in maceration of the infected tissues and thereby causing important economic losses. Our results show that co-suppressed potato plants lacking JAs are more susceptible to *D. dadantii* infection, similar to what has been reported for the Arabidopsis *aos* and *jar1* mutants (Fagard *et al.*, 2007; Antunez-Lamas *et al.*, 2009). These results suggest that a reduction in JA–Ile limits the capacity of the plant to mount a proper response to the pathogen, thus confirming that this hormone is a mediator of the plant defense response against *D. dadantii* (Fagard *et al.*, 2007). While the role of JAs in controlling inducible defense responses is well established, the involvement of these compounds in the development of constitutive defense barriers in plants has received less attention. Although characterization of several Arabidopsis mutants revealed the importance of the cell-wall composition in plant–pathogen interactions (Ellis *et al.*, 2002; Vogel *et al.*, 2002, 2004), the possibility that JAs control cell-wall composition remained largely unexplored. It has been reported that exogenous treatment with JA and ethylene leads to reduced cellulose synthesis and ectopic lignification in Arabidopsis (Cano-Delgado *et al.*, 2003). However, whether endogenously produced JAs control cell-wall composition was not known. We present evidence that JAs regulate cell-wall composition, and possibly its structure, during potato development in the absence of stress. In particular, basal JAs appear to reduce the methyl esterification of cell-wall pectins, most likely through activation of PME activity. Plant PMEs are usually encoded by large gene families (Pelloux *et al.*, 2007; Lionetti *et al.*, 2012), as is the case in potato. Moreover, PME activity is also modulated by PME inhibitors, whose presence may also be controlled by JAs (An *et al.*, 2009). A thorough characterization of these gene families is required to identify the direct molecular targets regulated by JAs, which is crucial information for advancing our mechanistic understanding of this process.

Importantly, the reduced levels of pectin methyl esterification fostered by JAs appear to have *in vivo* relevance for defense against pathogens, since they are targeted by *D. dadantii* PelA, PelB, PelC, PelD and PelE virulence factors to allow progression of soft rot disease. Indeed, pectate lyases are specific for pectins with a low degree of methyl esterification (Tardy *et al.*, 1997). Although PelA, PelB, PelC, PelD and PelE account for most of the pectate lyase activity, the *D. dadantii pel* strain lacking these five isozymes retains residual maceration activity on WT plants (Beaulieu *et al.*, 1993; this study). *D. dadantii* contains additional inducible pectate lyases (PelL, PelZ and PelX; Hugouvieux-Cotte-Pattat *et al.*, 1996; Shevchik *et al.*, 1999), which are considered as secondary macerating factors due to their low activity under standard conditions, and other pectinolytic enzymes such as pectin lyases, polygalacturonases and PMEs (Keen *et al.*, 1984; Tamaki *et al.*, 1988). This battery of enzymes is probably responsible for the high maceration ability of the *D. dadantii pel* mutant on *CoAOS1/2* plants. Their limited activity on WT cell wall is probably due to the different pattern and degree of pectin methyl esterification. The lower degree of pectin methyl esterification of WT plants facilitates Ca^{2+}-mediated cross-linking of the free carboxylic residues, which is necessary to form rigid 'egg-box' structures (Ridley *et al.*, 2001) that may render the cell wall less prone to degradation by these alternative pectinases. Another possibility is that the bacterial secondary pectinases are induced only during infection of *CoAOS1/2* plants, either because *CoAOS1/2* plants specifically produce an inductive signal, or because WT plants specifically produce a repressive signal. The alterations in the pectin matrix observed in *CoAOS1/2* plants may affect the production of cell wall-derived secondary signals that activate other downstream defense responses. Degradation of the cell wall upon infection releases elicitors whose nature depends on the composition of the cell wall itself, and on the accessibility of its components to the specific complement of pectolytic enzymes involved, and this variability may have physiological consequences. For example, it is known that oligogalacturonides act differently depending on the degree of polymerization and pattern of methyl esterification (Osorio *et al.*, 2008).

Our results suggest that JAs modify the composition and structure of the pectin matrix, rendering the tissues resistant to maceration by the *D. dadantii pel* strain. Our findings reveal a previously unrecognized connection between JAs, cell-wall synthesis and soft rot resistance in potato. It will be interesting to determine how this defense mechanism affects the interaction of potato with other pathogenic and non-pathogenic microorganisms.

EXPERIMENTAL PROCEDURES

Plant material

Potato plants (*Solanum tuberosum* cv. Désirée) were grown in the greenhouse at 22°C under a 16 h light/8 h dark photoperiod. Plants were transformed with *StAOS1* or *StAOS2* cDNAs under the control of the CaMV 35S promoter (Keil *et al.*, 1989). For generation of double transgenic plants, *StAOS2* cDNA under the control of the *StL700* promoter (Stockhaus *et al.*, 1987) was used for transformation. Potato plants were propagated and wounded as described previously (Royo *et al.*, 1999).

Arabidopsis thaliana (Col-0) and the *aos* mutant (Park *et al.*, 2002) were grown under a 14 h light/10 h dark photoperiod at

22°C. For over-expression of *StAOS1* and *StAOS2*, cDNAs were cloned into the pGWB2 vector (Nakagawa *et al.*, 2007) and transformed into plants by floral dipping (Clough and Bent, 1998). The *aos* mutant was crossed with *StAOS1* and *StAOS2* over-expression lines. Homozygous hygromycin-resistant plants were selected, and *aos* mutant plants were identified by PCR (Table S3). Ten-day-old *in vitro*-grown Arabidopsis seedlings were wounded by crushing approximately 50% of the above-ground tissues with a forceps.

Expression studies

RNA isolation and Northern analysis were performed as described previously (Royo *et al.*, 1996). Gene-specific DNA probes were amplified from cDNA clones using specific primers (Table S3). To determine the expression of *AtAOS*, a single-stranded riboprobe was synthesized according to standard procedures (Sambrook *et al.*, 1989). Protein extraction and Western blotting were performed as described previously (Farmaki *et al.*, 2007). Experiments were performed three times independently, yielding highly reproducible results.

In situ RNA hybridization

In situ RNA hybridization was performed on 8 μm transverse paraffin sections of potato leaves from wounded or control plants, using digoxigenin-labeled probes (Ferrandiz and Sessions, 2008). A 300 bp fragment from *StAOS1* and *StAOS2* cloned into the pGEM T Easy vector (Promega; www.promega.com) in both orientations was linearized and transcribed *in vitro* with T7 RNA polymerase to generate RNA antisense and sense digoxigenin-labeled riboprobes. A potato histone 4 (*H4*) antisense probe (374 bp) was used as a positive control (Figure S6).

Determination of endogenous OPDA, JA and JA–Ile levels from potato leaves

Hormone extraction and quantification were performed as described previously (Flors *et al.*, 2008). Before extraction, a mixture of internal standards containing 100 ng dihydrojasmonic acid and 100 ng prostaglandin B1 was added. Dry tissue (0.05 g) was immediately homogenized in 2.5 ml ultra-pure water (Millipore; www.millipore.com). After centrifugation (5000 ***g***, 40 min), the supernatant was acidified and partitioned against diethyl ether, dried, and resuspended in 1 ml water/methanol (90:10 v/v). A 20 μl aliquot of this solution was injected into a Waters Acquity UPLC system. The UPLC was interfaced with a triple quadrupole tandem mass spectrometer (Waters; www.waters.com). LC separation was performed using an Acquity UPLC BEH C18 analytical column (Waters) at a flow rate of 300 μl min^{-1}. Quantifications were performed using MassLynx 4.1 software (Waters) using internal standards (Sigma; www.sigmaaldrich.com) as a reference for extraction recovery and standard curves as quantifiers. Values are means and standard deviations from three biologically independent experiments. Data were statistically analyzed using two-way ANOVA with a significance level of $P < 0.05$.

Bacterial infection assays

Cultures of *D. dadantii* 3937 WT and the *pel* mutant were grown to late exponential phase ($OD_{600} = 1.0$) in NB liquid medium (Difco; www.bd.com), and then sub-cultured after 10-fold dilution into 30 ml fresh medium at 28°C to an OD_{600} of 0.60. Bacteria were harvested by centrifugation (4000 ***g*** for 15 min), washed twice in 10 mM $MgCl_2$ and immediately recovered by centrifugation (4000 ***g*** for 15 min). The resultant pellet was resuspended to an OD_{600} of 0.06 (3×10^7 cfu ml^{-1}) in the same buffer. All the steps were performed at room temperature. Nine plants of each line, kept at 28°C, 80% relative humidity and a 10 h light/14 h dark photoperiod, were inoculated using a syringe with 100 μl of a bacterial solution containing 3×10^6 cfu in the plant stem under the fifth leaf. Symptoms were recorded 2 days after inoculation, and the length of the stems showing maceration was measured. Data were statistically analyzed using Student's *t* test. Experiments were performed independently three times, yielding highly reproducible results.

Cell-wall analysis

FTIR analysis was performed using an insoluble homogenized cell-wall residue (AIR) obtained after treating leaves with methanol/chloroform (1:1 v/v) overnight, and then with acetone (Kacurakova *et al.*, 2000). FTIR spectra for each line were collected on a BRUKER IFS 66Vs spectrometer (Bruker Corporation; www.bruker optics.com). For each spectrum, 64 scans were co-added at a resolution of 4 cm^{-1} for Fourier transform processing and absorbance spectrum calculation using OMNIC software (Thermo Nicolet). Using WinDAS software, spectra were baseline-corrected, normalized and analyzed by PCA and the covariance matrix method (Kemsley, 1998).

AIR was de-starched using α–amylase (Sigma). After washing, total AIR was freeze-dried, and 10 mg ml^{-1} of material was treated with a combination of 0.1 units of PME from *Aspergillus aculeatus* (Novozymes; www.novozymes.com) and 0.3 units of endopolygalacturonase (endoPG) from *Aspergillus niger* (Megazyme; www.megazyme.com) or with endoPG alone, at 30°C in 50 mM ammonium formate, pH 5.5, with continuous agitation for 18 h. After enzymatic treatment, the suspension was centrifuged (16 000 ***g*** for 10 min at room temperature), and the supernatant with the solubilized oligosaccharides was dried.

For sugar analysis, 1 mg of each polysaccharide was hydrolyzed with 3 M trifluoroacetic acid (121°C, 1 h). After hydrolysis, the internal standard (*myo*-inositol, 100 μg) was added, and the hydrolysate was evaporated to dryness. The monosaccharides released were converted into their corresponding trimethyl-silyl-oximes (Rojas-Escudero *et al.*, 2004). The derivatives were analyzed by GLC (Bernabé *et al.*, 2011) in an Agilent 5975C instrument (www.agilent.com) equipped with an HP5–MS column and a flame ionization detector.

MALDI-TOF/MS was performed using pectic oligosaccharides obtained as described above. A 0.2 M stock solution of diammonium hydrogen citrate (DAHC) was prepared with ultra-pure water. The 2,5–dihydroxyacetophenone (DHAP) matrix stock solution was prepared by dissolving 10 mg DHAP in 1 ml of 50% acetonitrile. A DHAP/DAHC matrix solution was prepared by mixing DHAP stock solution with DAHC stock solution at a 1:1 ratio (v/v). Samples were diluted 1:8 v/v with DHAP/DAHC matrix solution, and a 1.0 μl aliquot of this mixture was manually deposited onto a 384-well OptiTOF™ plate (AB SCIEX; www.ABSciex.com), and allowed to dry at room temperature. Analyses were performed on an ABI-4800 MALDI TOF/TOF mass spectrometer (AB SCIEX) in reflector positive ion mode; the ion acceleration voltage was 25 kV. The detection mass range was set between 300 and 2000 *m/z*.

PME activity

For determination of PME activity, 1 g leaf tissue was homogenized in 50 mM Tris/HCl solution (pH 6.0) containing 1 M NaCl, and incubated at 4°C for 1 h. Extracts were centrifuged (13 000 ***g***, 15 min), and the supernatant was collected. A 10 μl sample (or

extraction buffer as blank) was added to 100 μl of 100 mM Tris/HCl (pH 7.5) containing 0.4 mg ml^{-1} pectin and 40 μl 3-Methyl-2-benzothiazolinone hydrazone (3 mg ml^{-1}). After addition of 10 μl alcohol oxidase (Sigma) at 0.01 units μl^{-1}, samples were incubated for 20 min at 30°C. Then 200 μl of a solution containing 5 mg ml^{-1} each of ferric ammonium sulfate and sulfamic acid were added. After 20 min at room temperature, 550 μl water was added to give a final volume of 1.0 ml, and the absorbance at 620 nm was determined (Anthon and Barrett, 2004). Data from three biologically independent experiments were statistically analyzed using one-way ANOVA with a significance level of $P < 0.05$.

Accession numbers

Sequence data may be found in the GenBank/EMBL/Solanaceae Genomics Resource databases under the accession numbers AJ457080 (*StAOS1*), AY135640 (*StAOS2*), X96406 (*StLOXH3*), AY135641 (*StAOC*), NM_001246944 (*SlOPR3*), BTO13697 (*SlJAR1*), AJ630505 (*StMYC2*), X03779 (*StPIN2*), X67846 (*StTD*), U50151 (*SlLAP*), PGSC0003DMT400078157 (*StH4*), At5g24770 (*AtVSP2*) and At5g42650 (*AtAOS*).

ACKNOWLEDGMENTS

We gratefully acknowledge Pilar Paredes, Tomás Cascón and Pedro Mario Rodríguez-Pascual for excellent technical assistance, the Proteomic Service (ProteoRed, Centro Nacional de Biotecnología) for MALDI-TOF/MS analysis, the gas chromatography service at Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, for sugar determination and the infrared spectroscopy service at the Instituto de Ciencia de Materiales de Madrid, Consejo Superior de Investigaciones Científicas for FTIR analysis. We thank Paul E. Staswick for providing *SlJAR1* cDNA and F. Vangijsegem for providing the *D. dadantii* 3937 *pel* mutant strain. This work was supported by grants from the Spanish Ministerio de Ciencia e Innovación (BIO2008-03052 to J.J.S.S., BIO2009-10784 to E.R., AGL-2009-12757 to E.L.S., BIO2008-04160 to S.P. and AGL2010-22300 to P.G.A.).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Expression analysis of the 13-LOX biosynthetic pathway and wound-responsive genes in potato leaves.

Figure S2. Characterization of single *CoAOS1* or *CoAOS2* potato plants.

Figure S3. Statistical analysis of FTIR data.

Figure S4. Identification of pectin oligosaccharides 868.42 and 842.70.

Figure S5. Oligosaccharide mass profiling of polygalacturonase-digested cell walls.

Figure S6. *In situ* hybridization control.

Table S1. Oxylipin profile upon wounding of transgenic potato plants specifically depleted of each *StAOS*.

Table S2. Neutral and acidic monosaccharide composition by trimethyl-silyl-oximes of cell walls obtained from transgenic and wild-type leaves.

Table S3. Primers used in this study and the restriction enzyme digestion approach used to generate specific probes.

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